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Free Radical Scavenging Activity of the Seed of *Phaseolus calcaratus* Roxburgh

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Abstract – The seed of *Phaseolus calcaratus* Roxburgh (PHCR) is traditionally used for anti-pyretic and anti-inflammatory effects. Although these effects are believed to be related to its antioxidant potential, little information is available for the mechanisms by which PHCR seed might scavenge free radicals or otherwise act as an antioxidant. In the present study, we purified some fractions from the ethanol extract of PHCR seed and evaluated each fraction's ability to scavenge free radicals generated by cell-free systems. We also identified active compound that is putatively responsible for free radical scavenging by analyzing NMR spectra. PHCR samples exhibited a concentration-dependent radical scavenging activity against hydroxyl radicals, superoxide anions, and DPPH radicals. Of the samples tested, a methanol-eluted sub-fraction from the PHCR extract, named FF_4, scavenged these radicals more effectively than the other fractions. We identified catechin-7-O-β-D-glucopyranoside as the active compound responsible for free radical scavenging potential of FF_4.
Keywords – free radical scavenging; catechin-7-O-β-D-glucopyranoside; *Phaseolus calcaratus* Roxburgh

Introduction

Bioactive materials having antioxidant potential can be clinically applied as preventive and therapeutic measures dealing with degenerative diseases caused by oxidative stress. There is a global trend toward using phenolic phytochemicals that are present in fruits, vegetables, oilseeds, and herbal plants as antioxidants and functional foods (Holst and Williamson, 2008; Donnez *et al*., 2009). *Phaseolus calcaratus* Roxburgh (PHCR), a type of azuki bean, is a common dietary component in China and Korea (Zhao *et al*., 2007). The seed of PHCR is used as a traditional anti-pyretic and anti-inflammatory medicine in Korea. Such medicinal effects are believed to be associated with its antioxidant potential. However, scientific basis showing the mechanisms by which PHCR seed scavenges free radicals is not clearly defined.

The antioxidant activity of a lot of bioactive constituents may be either direct, such as scavenging free radicals, or indirect, involving the chelation of transition elements (Lee *et al*., 1999; 2002). Moreover, these active constituents, which exhibit strong reducing activity, can also contribute to strong pro-oxidant activity (Halliwell *et al*., 1987; Laughton *et al*., 1989). In this study, we prepared several purified fractions from PHCR seed ethanol extract and evaluated their abilities to scavenge free radicals. The active compound that is putatively responsible for the antioxidant property of PHCR seed extracts is also identified by analyzing NMR spectra.

Experimental

Chemicals, plastics and antibodies – Unless otherwise specified, all of the chemicals, culture plastics, and antibodies were purchased from Sigma Chemical Co. (St. Louis, MO), Becton-Dickinson (Franklin Lakes, NJ), and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively.

Plant material – Dried PHCR seed was obtained from a traditional herbal market, located in Jeonju (Korea) and identified by Dr. H.K. Cho, director of Center for Health Care and Technology Development, HanPoong Pharmaceutical Co. Ltd. (Korea). A voucher specimen (HP-PHCRS) was deposited at the Center.

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Preparation of PHCR samples – In brief, dried seed (5.5 kg) was extracted with 50% ethanol by shaking at 20 ºC for 2 h three times. The extract was concentrated using a rotary vacuum evaporator (Rotavapor R110, Büchi, Switzerland) and lyophilized to yield a crude powder (756 g). The crude ethanol extract was suspended in distilled water and partitioned with the following stepwise solvents; *n*-hexane, ethyl acetate, *n*-butanol, and distilled water, which yielded four fraction samples (PCH, PCE, PCB, and PCW). The samples were concentrated and lyophilized to 28.1, 13.1, 65, and 520 g (3.7, 1.7, 8.6, and 68.8% of the initial amount) for PCH, PCE, PCB, and PCW, respectively. After determining antioxidant activity using cell-free reactive oxygen species (ROS) generating systems, the sample PCB was applied to Amberlite XAD-4 resin (mesh size 20-60; Sigma Chemical Co.) and diluted successively with distilled water, 30% methanol, 60% methanol, and 99% methanol to yield four fractions: distilled water (PCB1, 23.4 g), 30% methanol (PCB2, 13.6 g), 60% methanol (PCB3, 11.6 g), and 99% methanol (PCB4, 4.3 g). The PCB2 fraction was further separated into eight sub-fractions (F_1 to F_8) by chromatography using a Sephadex LH-20 column (83 cm × 2 cm, Amersham Pharmacia Biotech, Freiburg, Germany) eluted with 30% methanol. Based on the antioxidant activity-guided fractionation procedure, F_6 was again applied to the Sephadex LH-20 column and eluted with 60% methanol to obtain four fractions: FF_1 (0.028 g), FF_2 (0.029 g), FF_3 (0.2 g), and FF_4 (1.3 g).

Deoxyribose assay – The deoxyribose assay was conducted to determine the rate constants for reactions between either antioxidants or hydroxyl radicals (˙OH) (referred as non-site-specific scavenging assay) as described by Halliwell *et al* (1987). In brief, PHCR samples at different concentrations (0.01 - 1 mg) were mixed with 1 mL reaction buffer containing 100 μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM ascorbic acid (pH 7.4) before incubation for 1 h at 37 ºC. Subsequently, 1 mL of 0.5% 2-thiobarbituric acid in 0.025 M NaOH and 1 mL of 2.8% trichloroacetic acid were added to the mixtures and further incubated for 30 min at 80 ºC. After cooling the mixtures on ice, absorbance was measured at 532 nm using a spectrophotometer (Beckman, DU® 530, Germany). In addition, site-specific scavenging activity, which represented the ability of PHCR samples to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA only.

Ammonium thiocyanate assay – This assay was carried out to measure the antioxidant activity of PHCR samples against lipid peroxidation as described elsewhere (Takao *et al*., 1994). The substrate solution containing 400 μL of PHCR samples containing 0.01 to 2 mg, 200 μL of diluted linoleic acid (25 mg/mL 99% ethanol), and 400 μL of 50 mM phosphate buffer (pH 7.4) was incubated for 15 minutes at 40 ºC. A 100 μL aliquot was then mixed with a reaction solution containing 3 mL of 70% ethanol, 100 μL of ammonium thiocyanate (300 mg/mL distilled water) and 100 μL of ferrous chloride (2.45 mg/mL 3.5% HCl). After incubation at room temperature for 3 minutes, absorbance was measured at 500 nm.

Superoxide radical scavenging assay – Scavenging activity on superoxide radicals ($O_2^{\bullet -}$) was accessed by the method described by Gotoh and Niki with a slight modification (Gotoh and Niki, 1992). Briefly, different concentrations of PHCR samples were added to the reaction solution containing 40 μL of 30 mM EDTA (pH 7.4), 4 μL of 30 mM hypoxanthine in 50 mM NaOH, and 80 μL of 1.42-mM nitro blue tetrazolium (NBT). After incubation for 3 minutes, 30 μL of 0.5 U/mL xanthine oxidase was added to the mixture and the volume was brought up to 1 mL with 50 mM phosphate buffer (pH 7.4). The absorbance was measured at 560 nm.

DPPH scavenging assay – Scavenging activity on DPPH free radicals by PHCR samples was assessed according to the method reported by Gyamfi *et al* (1999). Briefly, 100 μL of the samples containing various amount of powdered extracts (0 - 100 μg) was mixed with 1 mL of 0.1 mM DPPH-ethanol solution and 450 mL of 50 mM Tris-HCl buffer (pH 7.4). After 30 minutes incubation at room temperature, reduction of DPPH free radicals was measured by reading the absorbance at 517 nm using a spectrophotometer.

Results

Antioxidant properties of PHCR samples on hydroxyl radicals and superoxide anions – We first measured the scavenging activity of PHCR samples on the hydroxyl radicals generated by Fe^{3+} ions and superoxide anions produced by the xanthine oxidase-mediated degradation of hypoxanthine (Fig. 1). The addition of the PHCR partitions prevented hydroxyl radicals from degrading the deoxyribose in a dose-dependent manner (Fig. 1A). Of the samples tested, PCE and PCB scavenged the hydroxyl radicals better than the other samples. We also determined the effects of hydroxyl radical scavenging by the PHCR samples using an ammonium thiocyanate assay to measure linoleic acid

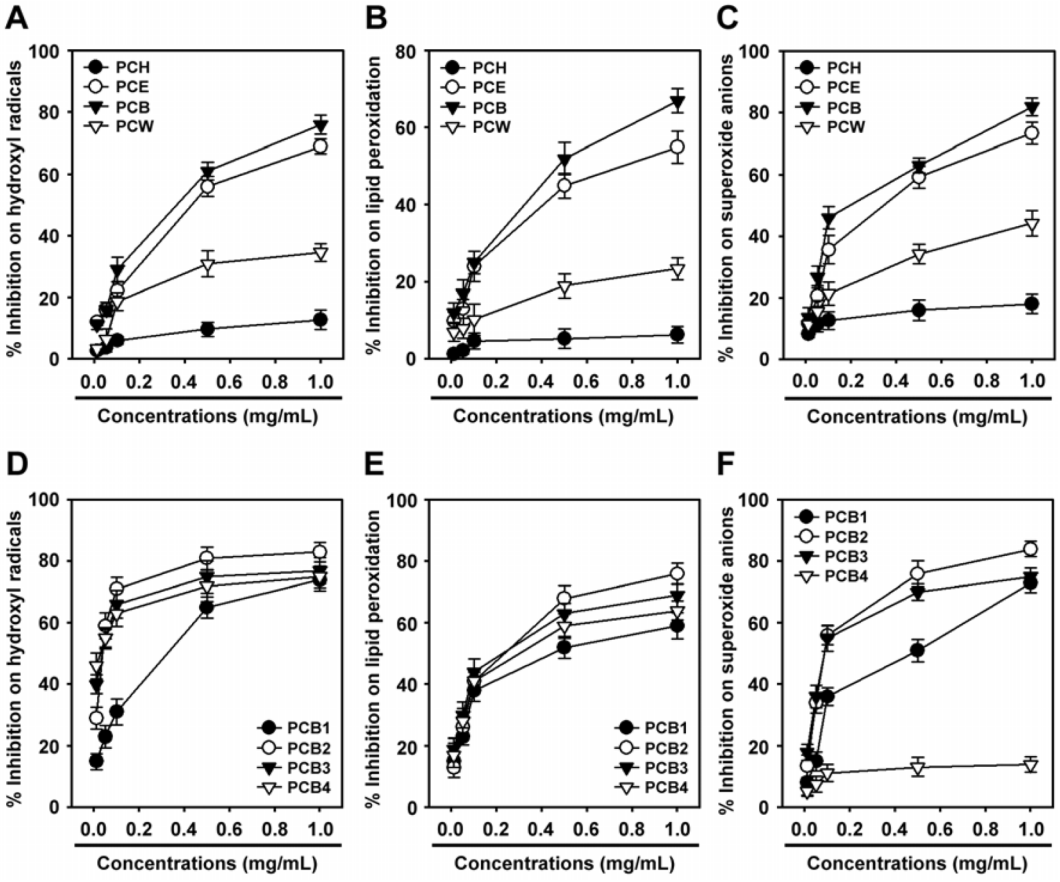

Fig. 1. The inhibitory effects of PHCR samples on the generation of hydroxyl radicals and superoxide anions.
Hydroxyl radicals were generated by Fenton's reaction using deoxyribose assay (A & D) and ammonium thiocyanate assay (B & E) systems, and the inhibitory effects of PHCR samples on hydroxyl radicals and lipid peroxidation are expressed as % inhibition. The inhibitory effect of PHCR samples was also tested by monitoring NBT reduction caused by superoxide anions using the hypoxanthine-xanthine oxidase system (C & F). The concentrations of PHCR samples tested ranged from 0.01 to 1 mg/mL. The results are the mean ± SD of three separate experiments.

oxidation. The samples inhibited Fe^{3+}-dependent linoleic acid oxidation, in which the inhibitory effects of PCB and PCE fractions were greater than other PHCR samples (Fig. 1B). In addition, PHCR samples inhibited NBT reduction efficiently, and the PCB fraction showed the most potent scavenging activity (Fig. 1C).

Based on the scavenging activity of PHCR partitions against hydroxyl radicals and superoxide anions, PCB was sub-fractioned and further analyzed for radical scavenging activity. Some of the PCB subfractions (PCB1 - 4) inhibited deoxyribose degradation (Fig. 1D), lipid peroxidation (Fig. 1E), and NBT reduction (Fig. 1F) more effectively than PCB itself. In particular, PCB2 showed the strongest potential to scavenge hydroxyl radicals and superoxide anions.

Effects of PCB2 and its subfraction, FF_4, on Fe^{3+}-dependent hydroxyl radical production and NBT reduction – PCB2 was subjected to Sephadex LH-20 column to obtain eight subfractions (F_1 to F_8). The subfraction, F_6, was further separated on the same column to prepare four fractions (FF_1 to FF_4). Of the FF_{1-4} samples, FF_4 showed the greatest antioxidant potential on the generation of hydroxyl radicals and superoxide anions under the same conditions (data not shown). We subsequently compared the ability of PCB2 and FF_4 on the prevention of hydroxyl radical-mediated lipid peroxidation. The FF_4 further inhibited Fe^{3+}-dependent linoleic acid oxidation in a dose-dependent manner. Greater sensitivity was noted in that approximately 50 μg/mL of FF_4 inhibited oxidation by 50% (Fig. 2A). We next determined whether the FF_4 fraction decreased hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radicals. The concentration-dependent inhibition of hydroxyl radical-induced deoxyribose degradation was observed in both site-specific and non-site specific assays. Greater antioxidant activity was

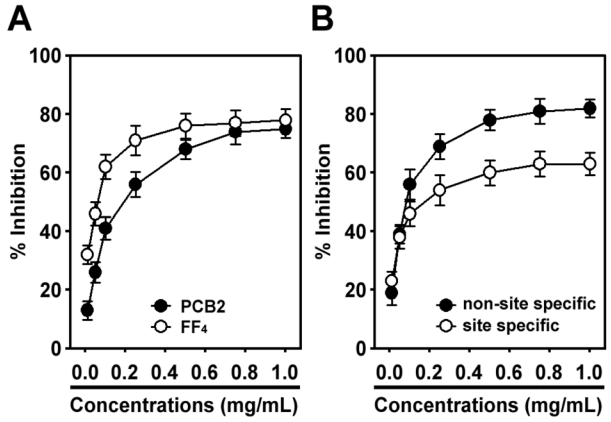

Fig. 2. The inhibitory effects of PCB2 and FF_4 on hydroxyl radical-mediated lipid peroxidation and deoxyribose degradation.

An ammonium thiocyanate assay was carried out in order to compare the inhibitory activity of PCB2 and FF_4 against lipid peroxidation (A). The non-site-specific and site-specific scavenging activities of FF_4 against hydroxyl radicals were also determined using deoxyribose assay system (B). The results are the mean ± SD of three separate experiments.

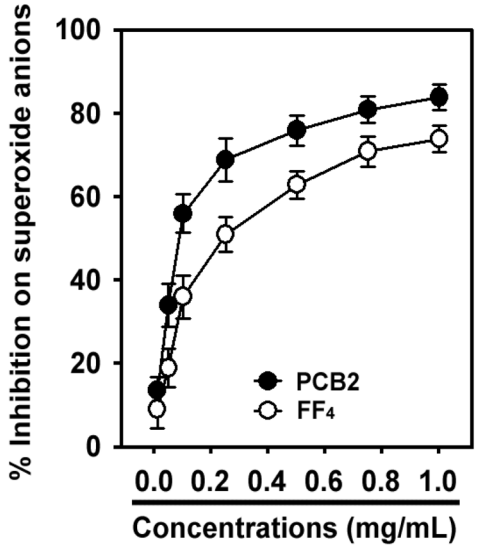

Fig. 3. The inhibitory effects of PCB2 and FF_4 on NBT reduction.

The scavenging activity of PCB2 and FF_4 on superoxide anions was compared using the hypoxanthine-xanthine oxidase system. The results are the mean ± SD of three separate experiments.

observed in the non-site specific assay, when the same FF_4 concentration was used (Fig. 2B). This finding suggests that the FF_4 fraction has greater potential to scavenge hydroxyl radicals directly than to chelate metal ions.

We also compared the scavenging activity of PCB2 and FF_4 on superoxide anions. Fig. 3 shows that both the PCB2 and the FF_4 samples inhibited NBT reduction

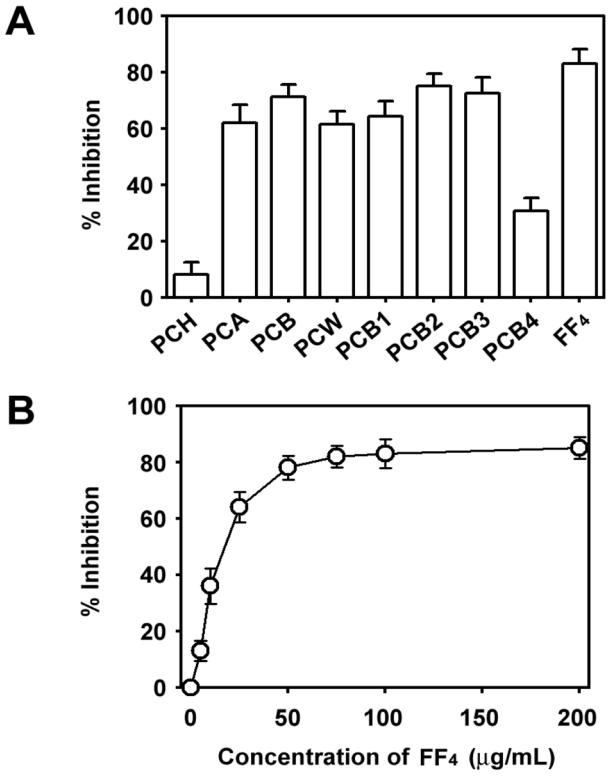

Fig. 4. The scavenging activity of PHCR samples on DPPH radicals.

(A) Direct scavenging activity of PHCR samples on DPPH radicals was determined. The results are expressed as % inhibition of the activity of 100 μg/mL of each sample. (B) In addition, dose-dependent scavenging activity of DPPH radicals by the FF_4 was examined, and the concentrations tested ranged from 5 to 200 μg/mL. The results are the mean ± SD of three separate experiments.

efficiently. However, the concentration of FF_4 required to achieve a 50% NBT reduction (IC_{50}), was 240 μg/mL, substantially higher than that of PCB2 (89 μg/mL). Either the PCB2 or the FF_4 fraction alone did not change the absorbance of the reaction solution containing only NBT, suggesting that the fractions did not directly reduce the NBT (data not shown).

Scavenging effects of PHCR fractions and FF_4 on DPPH radicals – PHCR partitions and PCB subfractions showed antioxidant potential to scavenge DPPH radicals (Fig. 4A). Among the samples tested, the FF_4 fraction scavenged DPPH radicals more efficiently than the other fractions. At a concentration of 100 μg/mL the FF_4 fraction almost completely scavenged the DPPH radicals. In contrast, PCH and PCB4 fractions inhibited only about 10% and 30%, respectively, of the DPPH radicals at the same concentrations. The calculated concentration of FF_4 required to scavenge 50% of DPPH radicals (IC_{50}) was 19 μg/mL (Fig. 4B).

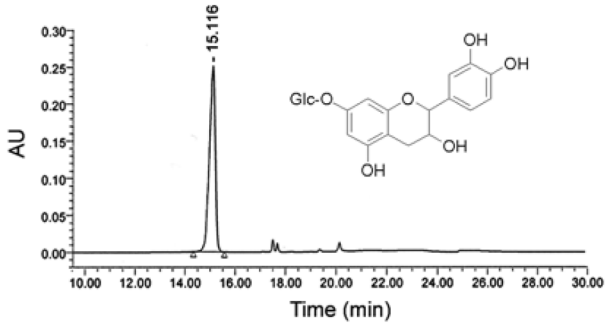

Fig. 5. HPLC chromatogram of FF_4.
HPLC was performed with a reverse-phase column (Shiseido Capcellpak C18 5 μm, 4.6 × 250 mm) eluted with a linear gradient of acetonitrile in 0.1% acetic acid. Column temperature was 30 ºC, injection volume 10 μL/time, and flow rate 1 mL/minute.

Identification of active compound in FF_4 – Since FF_4 appeared to exhibit the most efficient antioxidant activity, this fraction was applied to HPLC (Fig. 5). In the ^{1}H-NMR spectrum, the typical ABX system signals in aromatic proton at δ 6.75 (1H, d, J = 0.9 Hz, H-2'), δ 6.67 (1H, d, J = 7.8Hz, H-5'), δ 6.58 (1H, dd, J = 0.9, 7.8 Hz, H-6') were from 3, 4-hydroxyphenyl moiety of B-ring and the two hydroxyl bearing carbon signals of C-3, 4 were observed in downfield (δ 145.32, 145.31) compared with the peak of C-2, 5, 6 (δ 114.9, 115.6, 118.8) in C-NMR spectrum. These indicated that B-ring was a pyrocatechol moiety, which was substituted by hydroxyl group in C-3', 4'. Signals of two more aromatic protons, which were meta-coupled ones δ 6.07 (1H, d, J=1.2Hz, H-8) and δ 5.97 (1H, d, J=1.2Hz, H-6) were observed in ^{1}H-NMR spectrum, and the three carbon signals of C-5, 7 and 9 (δ 156.5, 157.2, 155.7) were deshelded and down field shifted rather than C-6, 8 and 10 (δ 96.6, 95.0, 102.3) in ^{13}C-NMR spectrum. These implied that the A-ring was related to a phlorglucinol moiety which was substituted by hydroxyl group in C-5, 7 and 9. Moreover, the proton signals of δ 4.54 (1H, d, J=7.2Hz, H-2), 3.85 (1H, m, H-3), 2.65 (1H, dd, J=2.4, 8.1Hz, H-4a), and 2.39 (1H, dd, J=2.4, 8.1Hz, H-4b) were observed in ^{1}H-NMR and the carbon signals in ^{13}C-NMR spectrum of C-2,3 and 4 (δ 81.5, 66.5, 28.0) indicated flavan-3ol moiety. Especially, the large coupling constant H-2 (J = 7.2Hz) in ^{1}H-NMR spectrum and carbon signals of C-2 at δ 81.1 in ^{13}C-NMR spectrum suggested a 2,3-*trans*-configuration (The *cis*-orientation between H-2 and 3 is accompanied with a broad singlet of H-2 in ^{1}H-NMR spectrum and carbon signals of C-2 at about δ 75.0 in ^{13}C-NMR spectrum).

At the monosaccharide moiety, an anomeric proton signal at δ 4.69 (d, J = 7.8Hz) and carbon signal at δ 100.9 (glc-1) including oxygenated methine and methylene carbons such as δ 77.3 (glc-3), δ 77.0 (glc-5), δ 73.6 (glc-2), δ 70.0 (glc-4) and δ 61.1 (glc-6) suggested the presence of a β-glucosylpyranoside (Wang *et al*., 2004; Lee *et al*., 2008). Thus the compound was presumed to be a catechin glycoside. To confirm the structure, we used the 2D-NMR technique. In the HMBC spectrum, owing to the correlation of the anomeric proton signals at δ 4.69 (glc-1) with the carbons at δ 157.2, which were assigned to C-7, the sugar moiety should be linked to C-7 of the catechin skeleton. In parallel with previous data (Wang *et al*., 2004; Benavides *et al*., 2006; Lee *et al*., 2008), all these spectral data revealed that the compound is catechin-7-O-β-D-glucopyranoside (Fig. 5).

Discussion

Hydroxyl radicals are known to be the most reactive of all reduced forms of dioxygen, and are thought to directly initiate cell damage (Rollet-Labelle *et al*., 1998). We used an Fe^{3+}-dependent system to test the antioxidant activity of the PHCR fractions, where hydroxyl radicals were generated via the Fenton reaction. The results of deoxyribose and ammonium thiocyanate experiments revealed that among PHCR samples, PCB2 was the most active fraction in scavenging hydroxyl radicals. This finding is evidenced by the dramatic inhibition of both the deoxyribose degradation and the linoleic acid oxidation (Fig. 1).

The superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems, either by auto-oxidation or enzymatic processes. The concentration of superoxide anions increases under conditions of oxygenative stress and related situations (Gotoh and Niki, 1992; Mates and Sanchez-Jimenez, 2000). Superoxide anions produce other kinds of cell-damaging free radicals and oxidizing agents (Liu and Ng. 2000). Therefore, we used a NBT assay system to evaluate the ability of PHCR samples to scavenge superoxide anions. These results were similar to those from the deoxyribose and the ammonium thiocyanate assays. The most active inhibition on NBT reduction was shown by PCB2 (Figs. 1C and F). These results suggested that PCB2 is a potential scavenger of superoxide anions, as well as hydroxyl radicals.

Next we tested the scavenging activities of PCB2 and its subfraction, FF_4, on hydroxyl radicals. The inhibition of FF_4 showed a greater sensitivity on lipid peroxidation than PCB2 (Fig. 2A). We also found that FF_4 strongly inhibited hydroxyl radical-induced deoxyribose degradation

in both site-specific and non-site-specific assays (Fig. 2B). In particular, FF_4 inhibited the deoxyribose degradation by the scavenging hydroxyl radicals directly rather than by chelating ferric ions. We did not observe FF_4 reducing the Fe^{3+}-EDTA complex, which represents the ability of FF_4 to stimulate hydroxyl radical generation in the deoxyribose assay system without ascorbic acid (data not shown). Further, the result from NBT assay suggested that FF_4 is a greater potential scavenger against hydroxyl radicals than superoxide anions (Fig. 3).

The indirect evidence of scavenging activity by FF_4 was further confirmed using a direct approach with DPPH radicals (Fig. 4), a stable radical used to evaluate the antioxidant activity of plant and microbial extracts (Hu and Kitts, 2000; Chang *et al*., 2001). In this assay, FF_4 exhibited the strongest scavenging activity against DPPH radicals, indicating that this fraction is a powerful antioxidant. This antioxidant activity was similar to those of other natural extracts or ascorbic acid (Lee *et al*., 2002).

We analyzed FF_4 to identify its active compounds that are antioxidant candidates. Chromatograms obtained from HPLC analysis showed only one main peak which revealed > 95% purity (Fig. 5). We finally identified catechin-7-O-β-D-glucopyranoside as the active compound of the fraction by analyzing ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectra and comparing them with published data (Wang *et al*., 2004; Benavides *et al*., 2006; Lee *et al*., 2008). Furthermore, the quantitative analysis using HPLC revealed that 8.458 mg of catechin-7-O-β-D-glucopyranoside was recovered from one gram of the dried PHCR seed.

In summary, oxidative stress occurs when the ROS produced exceeds the capacity of a biological system to eliminate them. This stress may play a role in several diseases, such as heart disease, degenerative neurological disease, and cancers (Trachtenberg and Hare, 2009; Hakim and Pflueger, 2010). In this study we show the potential of catechin-7-O-β-D-glucopyranoside to scavenge free radicals. This finding suggest strongly the capacity of this compound in preventing oxidative stress-mediated diseases, although further investigations will be needed.

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